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Effects of Watercress Consumption on Urinary Metabolites of Nicotine in Smokers[1]

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The effects of watercress consumption on the metabolism of nicotine in smokers were examined. Watercress is a rich source of phenethyl isothiocyanate (PEITC), an effective chemopreventive agent for cancers of the lung and esophagus induced in rodents by nitrosamines, including the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. PEITC is believed to inhibit nitrosamine carcinogenesis in rodents by inhibiting specific cytochrome P450 (P450) enzymes. Among the P450s involved in the activation of these nitrosamines are members of the 2A family. P450 2A6 is believed to be involved in the metabolism of both nicotine and its major metabolite cotinine. Therefore, we hypothesized that watercress consumption might inhibit nicotine and cotinine metabolism in smokers. The urine samples analyzed in this study were the same ones that we used in an earlier study (S. S. Hecht *et al.*, Cancer Epidemiol. Biomark. Prev., *4:* 877–884, 1995), in which we showed that watercress consumption increased levels of two metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone: NNAL and its glucuronide NNAL-Gluc. This increase was attributed either to inhibition of cytochromes P450 or induction of glucuronidation. In the present study, we quantified urinary nicotine and seven of its metabolites. There were no effects of watercress consumption on levels of nicotine, cotinine, *trans*-3'-hydroxycotinine, 4-oxo-4-(3-pyridyl)butanoic acid, or 4-hydroxy-4-(3-pyridyl)butanoic acid, indicating either that watercress ingestion has little effect on the oxidative metabolism of nicotine (presumably by P450 2A6 or other P450 enzymes) or that these enzymes are

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not important for nicotine and cotinine metabolism in smokers. However, watercress consumption resulted in a significant increase compared to baseline levels of the glucuronides of cotinine (25%, $P = 0.031$) and *trans*-3'-hydroxycotinine (33%, $P = 0.043$) during the period when it was consumed and in a nonsignificant increase in levels of the glucuronide of nicotine. These levels returned to baseline values after the watercress consumption period. There was a correlation between increases in levels of the glucuronides of *trans*-3'-hydroxycotinine and NNAL in the same subjects, suggesting the involvement of a common enzyme. Thus, the results of this study suggest that PEITC or another component of watercress induces UDP-glucuronosyltransferase activity in humans.

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Watercress (*Nasturtium officianale*) is an excellent source of the chemopreventive agent PEITC,[3] which occurs in this vegetable as its thioglucoside conjugate, gluconasturtiin (1, 2, 3) . When the watercress is chewed or cut, gluconasturtiin is hydrolyzed by the enzyme myrosinase, releasing PEITC. Based on studies of PEITC metabolites in human urine, a minimum of 2–6 mg of PEITC is released per ounce of watercress consumed (4 , 5) . PEITC is a very effective inhibitor of NNK-induced lung tumorigenesis in mice and rats (6, 7, 8, 9, 10, 11, 12) . It is also a strong inhibitor of NMBA-induced esophageal carcinogenesis in rats (13 , 14) . 3-Phenylpropyl isothiocyanate, a related naturally occurring isothiocyanate, inhibits esophageal tumorigenesis in rats treated with the tobacco-specific nitrosamine NNN (15) . In view of these data, PEITC is under investigation as a chemopreventive agent against tobacco-induced cancers, and a Phase I trial in smokers is currently in progress (16 , 17) .

In rodents, PEITC inhibits lung and esophageal tumorigenesis by inhibiting the metabolic activation of NNK in lung or NMBA in the esophagus (6 , 14 , 17, 18, 19, 20, 21, 22, 23) . This has been clearly demonstrated by quantitation of metabolites or DNA adducts in target tissues or cells (14 , 21, 22, 23) . PEITC is also an effective inhibitor of NNN metabolic activation in the esophagus (15) . P450s are involved in the metabolic activation of NNK, NMBA, and NNN in rodent lung and esophagus (24 , 25) . Whereas the specific P450 enzymes responsible for NNK, NMBA, and NNN metabolic activation in rat and mouse lung and rat esophagus are incompletely characterized at present, enzymes of the 2A family appear to play a role (24 , 25) .

Nicotine metabolism is outlined in Fig. 1⊞ (26 , 27) . Nicotine undergoes P450 catalyzed 5'-hydroxylation, yielding an iminium ion that is converted to cotinine. P450 2A6 appears to play a major role in nicotine 5'-hydroxylation (28 , 29) . Cotinine is further metabolized to *trans*-3'-hydroxycotinine, which is the most abundant urinary metabolite of nicotine (26 , 27) . P450 2A6 may also be important in catalysis of this reaction (30 , 31) . Nicotine, cotinine, and *trans*-3'-hydroxycotinine all are glucuronidated (26 , 27) . Another substantial pathway of nicotine/cotinine metabolism is formation of keto acid and hydroxy acid (32) . Nicotine and these seven urinary

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metabolites account for greater than 90% of the nicotine dose (26 , 32) .

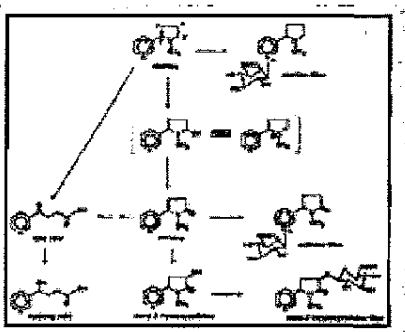

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Fig. 1. Overview of major pathways of mammalian nicotine metabolism.

We have previously shown that watercress consumption modifies the metabolism of NNK in smokers (5) . Upon consumption of watercress, urinary levels of the NNK metabolites NNAL and its *O*-glucuronide (NNAL-Gluc) increased. This was attributed either to inhibition of the α-hydroxylation metabolic activation pathways of NNK and NNAL or to induction of NNAL glucuronidation. P450s involved in the metabolism of NNK in humans include P450 1A2, 2A6, and 3A4 (24) . *In vitro*, PEITC inhibits P450 1A2 mediated NNK metabolic activation (33) . Other studies indicate that watercress consumption inhibits P450 2E1 activity in humans (34 , 35) .

The studies described above indicate the common involvement of P450 2A enzymes in the metabolism of nicotine, NNN, and NNK. There may also be common glucuronidation pathways. Because watercress consumption affected NNK metabolism, we hypothesized that it may also perturb nicotine metabolism. Therefore, in this study, we investigated the effects of watercress consumption on nicotine and cotinine metabolism, using the same urine samples as in our original study of NNK metabolism in smokers who consumed watercress.

Recently, we have developed methods to quantify human urinary hydroxy acid and keto acid (Fig. 1 ; Ref. 32) . Another goal of the present study was to compare urinary levels of these metabolites to those of nicotine and the other five nicotine metabolites shown in Fig. 1 to quantify more completely nicotine metabolism in smokers.

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Urine Samples.

These were saved from our previous study of the effects of watercress consumption on NNK metabolism in smokers (5) . Twelve subjects participated; urine samples from 11 were analyzed in the previous study. The

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protocol from that study is outlined in Fig. 2. Briefly, each subject served as his or her own control. In the baseline period (period 1), 24-h urine samples were collected for 3 consecutive days. In the watercress consumption period (period 2), which commenced 1–4 days later, each subject consumed 2 OZ. (56.8 g) of watercress at each meal for 3 days. Twenty-four h urine samples were collected beginning with the first morning void on each of the three watercress consumption days. (In the previous study, a 24-h urine sample was also collected on the day following watercress consumption, but that was not analyzed here.) Follow up periods occurred 1 (period 3) and 2 (period 4) weeks after watercress consumption; 24-h urine samples from these periods were available for 10 of the 12 subjects.

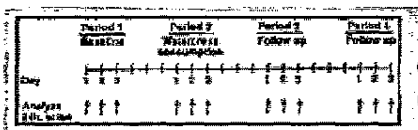

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Fig. 2. Protocol for examining the effects of watercress consumption on nicotine metabolism in smokers. Number of days between periods varied. See "Patients and Methods" and Hecht *et al.* (5) for further details.

Each subject smoked a constant number of cigarettes (±3) throughout the study. They also abstained from eating cruciferous vegetables, with the exception of the watercress consumption days.

Analysis of Urine.

Samples were analyzed for free cotinine and nicotine by gas chromatography-mass spectrometry as described previously (36) . [*methyl*-D_3]cotinine and [*methyl*-D_3]nicotine were used as internal standards. Total cotinine and nicotine were assayed by treating the samples with 0.1 N NaOH for 30 min at 70°C to release the aglycones prior to analysis for cotinine and nicotine. The levels of cotinine-Gluc and nicotine-Gluc were determined by calculating the difference between total and free concentrations of cotinine and nicotine. Previous studies have shown that this difference is due to the pyridine-*N*-glucuronides of cotinine and nicotine (26 , 27) . The level of *trans*-3'-hydroxycotinine was analyzed as its *tert*-butyldimethylsilyl derivative as described previously (37) , using *cis*-3'-hydroxycotinine as internal standard. Analysis was by gas chromatography-mass spectrometry with selected ion monitoring for *m/z* 249, loss of the *tert*-butyl group. *trans*-3'-Hydroxycotinine-Gluc present in urine was hydrolyzed by treating the samples with ß-glucuronidase. Samples were then analyzed for free *trans*-3'-hydroxycotinine as above, and the level of *trans*-3'-hydroxycotinine-Gluc was calculated. All samples were analyzed in duplicate; the two determinations agreed within 5%. The limit of detection was 1–2 ng/ml urine.

Keto acid and hydroxy acid were analyzed as described; the data in Table 1 are single determinations (32) . Creatinine was assayed by Fairview University Medical Center Diagnostic Laboratories (Minneapolis, MN) using Vitros CREA slides.

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View this table: [in this window] [in a new window] Table 1 Urinary nicotine metabolites (μmol/24 h) in smokers who consumed watercress[a]

Statistical Analysis.

Data from each subject were averaged over the days of each period. Paired *t* tests on these averages, as well as the Wilcoxon signed rank test, were used to determine the effects of watercress consumption on the metabolites.

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Characteristics of the study subjects were described previously (5) . Briefly, there were six males and six females, with a mean age of 30.2 ± 7.1 (SD) years. They smoked 15.0 ± 3.3 (SD) cigarettes per day. The mean estimated minimum amount of PEITC ingested on the watercress consumption days, based on analysis of its major metabolite in urine, was 37 mg (5) . Metabolites of PEITC were not detected on the other days of the study. Diet records indicated that all subjects complied with the protocol and avoided cruciferous vegetables or other sources of isothiocyanates during the study, except on the watercress consumption days.

The effects of watercress consumption on urinary nicotine metabolites are summarized in Table 1. Watercress consumption had no significant effect on urinary levels per 24 h of nicotine, cotinine, *trans*-3'-hydroxycotinine, keto acid, or hydroxy acid. However, levels of both cotinine-Gluc and *trans*-3'-hydroxycotinine-Gluc increased significantly during the watercress consumption period. For cotinine-Gluc, the overall increase was 25% ($P = 0.031$), whereas for *trans*-3'-hydroxycotinine-Gluc, the overall increase was 33% ($P = 0.0043$). Levels of nicotine-Gluc also increased during the watercress consumption period, but this increase was not significant. Similar results were obtained when the data were expressed per mg of creatinine (data not shown). The increases in cotinine-Gluc and *trans*-3'-hydroxycotinine-Gluc in the watercress consumption period compared to the baseline period were also significant when analyzed by the Wilcoxon signed rank test. Levels of cotinine-Gluc, *trans*-3'-hydroxycotinine-Gluc, and nicotine-Gluc decreased in periods 3 and 4 after cessation of watercress consumption. However, these levels were not significantly different from those in the watercress consumption period. Overall changes in levels of cotinine-Gluc, *trans*-3'-hydroxycotinine-Gluc, and nicotine-Gluc are summarized in Fig. 3.

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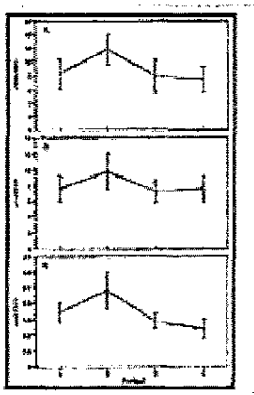

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Fig. 3. Mean levels ± SE of *trans*-3'-hydroxycotinine-Gluc (*A*), cotinine-Gluc (*B*), and nicotine-Gluc (*C*) in the urine of smokers before watercress consumption (period 1), during the 3 days of watercress consumption (period 2), and after watercress consumption (periods 3 and 4). Data are from Table 1.

We also carried out the same comparisons using data from days 2 and 3 only of the watercress consumption period, excluding day 1 because these urine samples include nicotine metabolites from the previous 24-h period in which watercress was not consumed. The results of this analysis were similar to those described above. Significant increases in levels of cotinine-Gluc ($P = 0.041$) and *trans*-3'-hydroxycotinine-Gluc ($P = 0.003$) were observed compared to the baseline period. Nicotine-Gluc also increased, but not significantly. All three glucuronides decreased after cessation of watercress consumption. The decrease was significant in period 4 compared to the watercress consumption period for nicotine-Gluc ($P = 0.049$) and nearly significant for *trans*-3'-hydroxycotinine-Gluc ($P = 0.072$).

Levels of *trans*-3'-hydroxycotinine-Gluc or cotinine-Gluc inurine during the 3 days of watercress consumption were compared to baseline levels, and the percentage of change for each subject was calculated. There was considerable interindividual variation among the 12 subjects, as illustrated in Fig. 4. For *trans*-3'-hydroxycotinine-Gluc,5 of the 12 subjects had relatively large increases, ranging from 71 to 217%, whereas 6 had more modest increases, and 1 subject showed a decrease. For cotinine-Gluc, the increases were generally smaller, with four subjects having increases of 45% or greater, whereas two individuals showed decreases. There was a nonsignificant ($r = 0.54$; $P = 0.067$) correlation between changes in *trans*-3'-hydroxycotinine-Gluc and cotinine-Gluc.

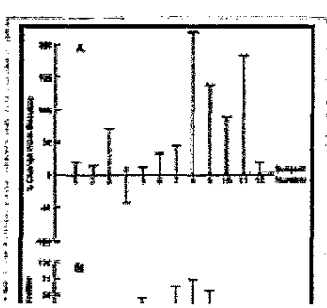

Fig. 4. The percentage change in 24-h urinary levels of *trans*-3'-hydroxycotinine-Gluc (*A*) and cotinine-Gluc (*B*) in each subject during the 3 days of watercress consumption (period 2) *versus* baseline (period 1). Percentage change was based on mean values for the 3 days of each period.

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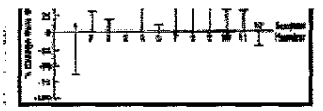

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Correlations among four glucuronide metabolites at baseline are summarized in Table 2. NNAL-Gluc correlated significantly with *trans*-3'-hydroxycotinine-Gluc but not with cotinine-Glucor nicotine-Gluc. Levels of *trans*-3'-hydroxycotinine-Gluc correlatedsignificantly with those of cotinine-Gluc.

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Correlations among levels of four glucuronides in 11 smokers[a]

We then compared the percentage changes in levels of *trans*-3'-hydroxycotinine-Glucand cotinine-Gluc resulting from watercress consumption to the corresponding changes in NNAL-Gluc levels, as determined in our previous study (5) . There was a correlation between the changes in *trans*-3'-hydroxycotinine-Gluc and NNAL-Gluc among the 11 subjects for whom data were available ($r = 0.63$; $P = 0.035$). These variables were also correlated when only the last 2 days of watercress consumption were compared to baseline ($r = 0.71$; $P = 0.015$), as illustrated in Fig. 5. Changes in NNAL-Gluc did not correlate with changes in cotinine-Gluc ($r = 0.42$; $P = 0.20$).

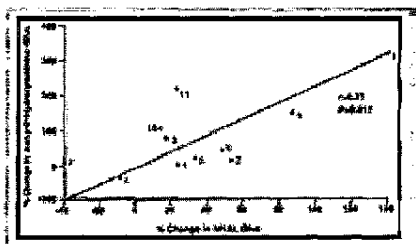

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Fig. 5. Correlation between percentage change in *trans*-3'-hydroxycotinine-Gluc and NNAL-Gluc in 10 subjects in the last 2 days of the watercress consumption period (period 2) versus the 3 baseline days (period 1). The identifying number of each subject is indicated.

Levels of nicotine and the seven metabolites quantified in this study, as a percentage of total quantified urinary metabolites, are presented in Table 3, using data from the baseline period for the 12 subjects. The major urinary metabolite was *trans*-3'-hydroxycotinine,which, together with its glucuronide, accounted for more than one-half of the quantified metabolites. Cotinine plus cotinine-

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Gluc comprised about one-quarter of the total, whereas nicotine plus nicotine-Gluc and hydroxy acid plus keto acid each accounted for about 10%.

View this table: Table 3 Average urinary excretion of nicotine and metabolites as percentage of nicotine and seven quantified metabolites[a]
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The results indicate that watercress consumption had no effect on urinary levels of nicotine, cotinine, and *trans*-3'-hydroxycotinine. Therefore, either watercress consumption has a minimal effect on P450 2A6 or this enzyme is not the major one involved in nicotine and cotinine metabolism. Although the role of P450 2A6 *versus* other P450s in nicotine and cotinine metabolism in smokers has not been quantified, the available data indicate that P450 2A6 is important (28, 29, 30, 31) . This suggests that the effect of watercress consumption on P450 2A6 may be small.

In contrast to the null effects of watercress consumption on levels of unconjugated nicotine metabolites, we observed significant increases in levels of cotinine-Gluc and *trans*-3'-hydroxycotinine-Gluc. These results indicate that watercress consumption induces glucuronidation of these nicotine metabolites in humans. It is unclear whether this is due to released PEITC or to other constituents of watercress. As glucuronidation is generally a detoxification mechanism, this observation could be important with respect to the development of chemopreventive agents.

In our previous study, the effects of watercress consumption on NNK metabolism were examined using the same urine samples as analyzed here. We found that there was a significant increase in urinary NNAL-Gluc levels, as well as NNAL plus NNAL-Gluc, during the second 2 days of watercress consumption, compared to baseline. It was not possible to determine whether the increase in NNAL-Gluc was due to an increase in available substrate (NNAL) or to induction of glucuronidation. We hypothesized that watercress consumption inhibited P450 1A2, which is known to be involved in hepatic α-hydroxylation of NNK (33) . This is reasonable because *in vitro* studies have shown that PEITC inhibits NNK metabolism by P450 1A2 (33) . Inhibition of α-hydroxylation would lead to higher levels of urinary NNAL. The results of the present study strongly suggest that glucuronidation of NNAL may have been induced. Increases in levels of NNAL-Gluc correlated with increases in *trans*-3'-hydroxycotinine-Gluc (Fig. 5) . Presently, there is no published information on the specific UDP-glucuronosyltransferase enzymes responsible for glucuronidation of NNAL and *trans*-3'-hydroxycotinine, but our data suggest that the same enzyme may be involved. The correlation between levels of NNAL-Gluc and *trans*-3'-hydroxycotinine-Gluc at baseline also support

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the involvement of a common enzyme.

Studies of the effects of PEITC on glucuronidation enzymes in rats are limited and provide little evidence supporting induction. Guo *et al.* (38) treated rats with 1 mmol of PEITC per kg of body weight, by gavage, and assessed liver, lung, and nasal mucosa microsomal UDP-glucuronosyltransferase activity with 4-nitrophenol as substrate. The liver activity was slightly decreased 2 h after treatment and then returned to baseline levels 6–24 h after treatment and was slightly increased at 48 h. There were no effects on activities in lung and nasal mucosa. Staretz *et al.* (23) investigated hepatic microsomal NNAL glucuronidation in rats given dietary PEITC (3 μmol/g diet, the dose used for chemoprevention) for 4, 12, or 20 weeks. There was no effect of PEITC. However, it should be noted that the major glucuronide of NNAL detected in rat urine is (*S*)-NNAL-Gluc, whereas in human urine, the predominant form is (*R*)-NNAL-Gluc (39 , 40) . Therefore, different forms of UDP glucuronosyl transferases may be involved in rat and human glucuronidation of NNAL.

There have been two previous reports on the effects of watercress consumption on drug glucuronidation in humans. In one, Chen *et al.* (34) found no effect on glucuronidation of acetaminophen. The other is our previous investigation of the effects of watercress consumption on NNK metabolism (5) , which is discussed above. Brussels sprouts or cabbage consumption resulted in increased glucuronidation of phenacetin but not oxazepam (41) . These results are consistent with the presence in these vegetables of glucobrassican, which is hydrolyzed to indole-3-carbinol during chewing (2 , 3) . Indole-3-carbinol is a known inducer of glucuronidation in rats and mice (42 , 43) . There is no evidence that watercress contains glucobrassican, although cabbage does contain relatively small amounts of gluconasturtiin, the precursor to PEITC (2 , 3) .

Thus, the limited data available to date preclude description of the mechanism by which watercress consumption increases glucuronidation of nicotine metabolites. PEITC or other constituents of watercress may induce specific UDP-glucuronosyl transferases. It is also possible that the balance of glucuronidation *versus* hydrolysis of glucuronides by gut ß-glucuronidase may be affected by a constituent of watercress (44) .

A second goal of this study was to profile the urinary metabolites of nicotine in smokers. In particular, we wanted to determine the concentrations of hydroxy acid and keto acid relative to other metabolites. We recently developed a method to quantify hydroxy acid and keto acid in human urine, but limited data were available concerning their concentrations relative to those of other nicotine metabolites (32) . The results summarized in Table 3 demonstrate that hydroxy acid is the fifth most abundant nicotine metabolite in our smokers' urine, whereas keto acid is the least abundant of those studied here. Our data are in good agreement with two previous studies of nicotine metabolites in smokers' urine (26 , 27) . All studies concur that *trans*-3'-hydroxycotinine is the major urinary metabolite of nicotine. This is followed by cotinine and cotinine-Gluc; in our study, cotinine slightly exceeded cotinine-Gluc, whereas in the other studies, the reverse was observed. Levels of nicotine

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and *trans*-3'-hydroxycotinine-Gluc ranged from about 8 to 10% in all studies. We found that hydroxy acid levels were about the same as those of nicotine. The levels of all other metabolites were lower than those of hydroxy acid. Hydroxy acid and keto acid are likely formed by the same pathway. McKennis *et al.* proposed that these metabolites are produced during metabolism of cotinine (45, 46, 47) . We have proposed that they may be formed by mammalian 2'-hydroxylation of nicotine (32) . If this hypothesis is correct, then the extent of 2'-hydroxylation of nicotine in smokers, leading to hydroxy acid and keto acid, would be 10–15% of that of 5'-hydroxylation, which ultimately produces *trans*-3'-hydroxycotinine, *trans*-3'-hydroxycotinine-Gluc, cotinine, cotinine-Gluc, and cotinine-*N*-oxide.

A difference between our results and those of Benowitz *et al.* (26) concerns the relationship of cotinine-Gluc and *trans*-3'-hydroxycotinine-Gluc. They found no correlation between the levels of these two metabolites, whereas they were strongly correlated in our study. The involvement of various UDP-glucuronosyl transferases in nicotine metabolism requires further study.

In summary, the results of this study demonstrate that watercress consumption perturbs nicotine metabolism by significantly increasing glucuronidation of cotinine and *trans*-3'-hydroxycotinine and that the latter correlates significantly with glucuronidation of NNAL. It is not clear whether this is due to PEITC or to another constituent of watercress. No other significant effects on nicotine metabolism were observed, suggesting that watercress consumption has little effect on oxidative nicotine metabolism by P450 2A6 or related enzymes. The relative levels of seven urinary nicotine metabolites were also established in this study.

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Footnotes

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[2] To whom requests for reprints should be addressed, at Box 806 Mayo, 420 Delaware Street SE, Minneapolis, MN 55455.

[3] The abbreviations used are: PEITC, phenethyl isothiocyanate; cotinine-Gluc, *N*-ß-D-

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glucosiduronosyl-(*S*)-(-)-cotinine inner salt; hydroxy acid, 4-hydroxy-4-(3-pyridyl)butanoic acid; *trans*-3'-hydroxycotinine-Gluc, *trans*-3'-hydroxycotinine-ß-*O*-D-glucosiduronic acid; keto acid, 4-oxo-4-(3-pyridyl)butanoic acid; nicotine-Gluc, *N*-ß-D-glucosiduronosyl-(*S*)-(-)-nicotine inner salt; NMBA, *N*-nitrosomethylbenzylamine; NNAL, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol; NNAL-Gluc, [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]ß-*O*-D-glucosiduronic acid; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*'-nitrosonornicotine; P450, cytochrome P450.

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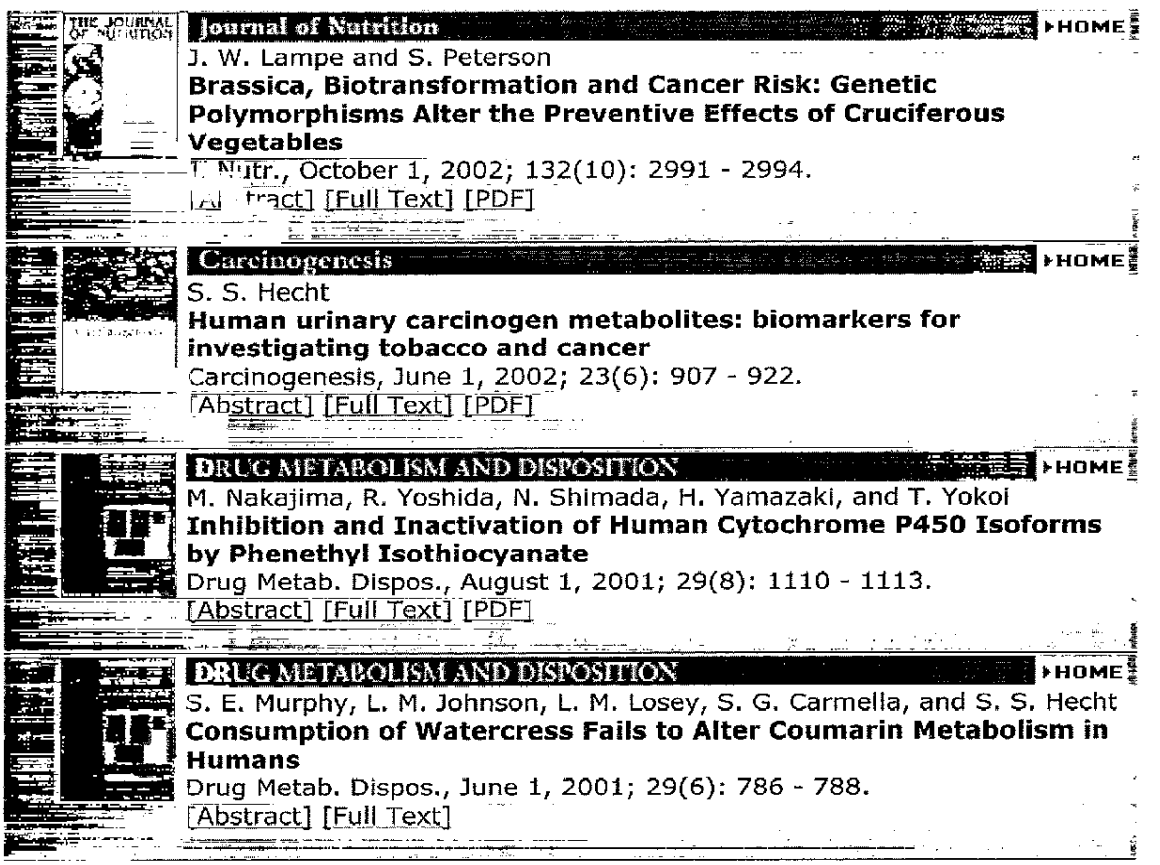

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